AMENDMENTS TO THE SPECIFICATION

Between the Title and the first paragraph, please insert the following section heading and paragraph:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is the National Stage of International Application Number PCT/US2004/019766, filed June 21, 2004, which claims the benefit of U.S. Provisional Application No. 60/480,161, filed June 20, 2003.

Please amend and replace paragraph [0022] with the following paragraph:

Fig. 6 demonstrates that the TTSWSQCSKS **(SEQ ID NO: 1)** sequence in T1 contains critical determinants for $\alpha_6\beta_1$-dependent cell adhesion. Site-directed alanine substitutions of the T1 sequence in the T1-GST fusion protein were performed as described in Materials and Methods. Wild type T1 fusion protein (GST-T1-WT), its scrambled variant (GST-T1-Scram) or the alanine substituted mutants was coated onto microtiter wells at a protein concentration of 200 μg/ml. After blocking with 1% BSA, fibroblast adhesion proceeded as described. Results are means ± S.D. of triplicate determinations and are representative of three experiments.

Please amend replace paragraph [0025] with the following paragraph:

Fig. 9 depicts the construction and expression of CCN1 and mutants. A, schematic diagram of constructs of wild type CCN1 (WT) and mutants either bearing the K239E point mutation in T1 (SM), disruptions in H1 and H2 (DM), or combined mutations in T1, H1, and H2 (TM). Each construct is similarly endowed with an N-terminal secretory signal and a C-terminal FLAG epitope tag. Recombinant proteins were expressed in insect cells via a baculovirus vector. Wild type T1, H1, and H2 sequences and specific a.a. changes in the mutants are shown. B, fibroblasts were plated on microtiter wells coated with either GST, GST-T1 peptide fusion, or GST-T1 (K239E) peptide fusion protein ~~(50 ①g/ml each)~~ **(50 µg/ml each).** Cells were allowed to adhere at 37°C for 20 min. After washing, adherent cells were fixed, stained with methylene blue, and quantified by absorbance at 620 nm. C, Coomassie brilliant blue stained 10% SDS-PAGE in which FLAG affinity-purified recombinant proteins (2 µg each) were electrophoretically separated. Molecular mass (kDa) of markers are

shown at left. The gel was immunoblotted with polyclonal anti-CCN1 antibodies and shown in the lower panel.

Please amend and replace paragraph [0033] with the following paragraph:

CCN1 is an angiogenic inducer that plays an essential role in normal vascular development during embryogenesis. *See* Mo *et al.* (2002) *Mol. Cell Biol.* **22,** 8709-8720. *See also* U.S. Pat. No. 6,413,735, and U.S. Application Serial No. 09/495,448 (allowed), both incorporated herein by reference. We have recently shown that the proangiogenic activities of CCN1 are mediated through integrins $\alpha_6\beta_1$ and $\alpha_v\beta_3$ in unactivated and activated HUVECs, respectively. *See* Leu *et al.* (2002) *J. Biol. Chem.* **277,** 46248-46255. In addition to integrin $\alpha_6\beta_1$ interaction with the T1 sequence in the TSP1 domain of CCN1, adhesion of fibroblasts and unactivated endothelial cells to CCN1 also requires heparin sulfate proteoglycans to act as co-receptors which interact with the heparin binding motifs in the CCN1 C-terminal domain. *See* Chen *et al.* (2000) *J.Biol.Chem.* **275,** 24953-24961. In this study, we have employed functional and biochemical analyses to define a 17-residue T1 sequence (GQKCIVQTTSWSQCSKS) **(SEQ ID NO: 2)** in the CCN1 domain III as a novel integrin $\alpha_6\beta_1$ binding site. We have also determined that heparin binding sites H1 and H2 are influential in the activity of the $\alpha_6\beta_1$-HSPG coreceptor complex in functions such as cell adhesion that are an important part of angiogenesis. These findings provide a basis for the development of $\alpha_6\beta_1$ antagonists and a target for mutational analyses to examine the role of integrin $\alpha_6\beta_1$-CCN1 interaction in angiogenesis.

Please amend and replace paragraph [0040] with the following paragraph:

By alanine substitution mutagenesis of the T1-GST fusion protein, we showed that the C-terminal portion of T1 (TTSWSQCSKS) **(SEQ ID NO: 1)** contains critical determinants for $\alpha_6\beta_1$-dependent cell adhesion. Of note is the double T231A/T232A and W234A/K239A substitutions that result in complete loss of its capacity to support cell adhesion. This 10-residue segment is highly conserved among other CCN family members with only two non-conserved substitutions among CCN1, CCN2 and CCN3. Therefore, it is conceivable that $\alpha_6\beta_1$ also binds to the corresponding T1 sequences in other CCN proteins. Consistent with this notion, soluble T1 peptide also inhibits $\alpha_6\beta_1$-dependent fibroblast adhesion to CCN2 and CCN3. These results lead us to conclude that the conserved TT*X*WS*X*CSKS **(SEQ ID**

NO: 3) sequence (*X* represents a non-conserved residue) in CCN proteins defines a novel recognition motif for integrin $\alpha_6\beta_1$. An important feature of this sequence is that any single alanine substitution of the conserved residues (i.e., T232A, W234A, S235A, S238A, and K239A) results in a drastic loss of $\alpha_6\beta_1$ binding activity, suggesting that it requires multiple coordination interaction with the ligand binding pocket in integrin $\alpha_6\beta_1$.

Please amend and replace paragraph [0041] with the following paragraph:

Integrin $\alpha_6\beta_1$ has a limited ligand spectrum that includes laminin, CCN proteins, invasin, fertilin and a collagen fragment known as tumstatin. *See* Sonnenberg *et al.* 1990) *J. Cell Biol.* **110,** 2145-2155; Maeshima *et al.* (2001) *J Biol.Chem.* **276,** 15240-15248; Isberg *et al.* (1990) *Cell* **60,** 861-871; Almeida *et al.* (1995) *Cell* **81,** 1095-1104. These diverse $\alpha_6\beta_1$ ligands that are involved in various biological processes are not structurally related. Several $\alpha_6\beta_1$ binding sequences have been identified by screening synthetic peptides derived from some of these $\alpha_6\beta_1$ ligands. These include the NPWHSIYITRFG **(SEQ ID NO: 4)** and TWYKIAFQRNRK **(SEQ ID NO: 5)** sequences from the laminin a1 chain. *See* Sonnenberg *et al.* 1990) *J. Cell Biol.* **110,** 2145-2155; Nomizu *et al.* (1995) *J. Biol. Chem.* **270,** 20583-20590; Nakahara *et al.* (1996) *J. Biol. Chem.* **271,** 27221-27224. In addition, TDE-containing peptides from the disintegrin domain of the fertilin b subunit disrupt sperm-egg fusion presumably by blocking integrin $\alpha_6\beta_1$-fertilin interaction. *See* Myles *et al.* (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91,** 4195-4198. Several other $\alpha_6\beta_1$ binding peptides have also been isolated by screening phage display and synthetic peptide combinatorial libraries; however, these sequences are not present in any known $\alpha_6\beta_1$ ligand. *See* Murayama *et al.* (1996) *J.Biochem. (Tokyo)* **120,** 445-451; Pennington *et al.* (1996) *Mol.Divers.* **2,** 19-28; DeRoock *et al.* (2001) *Cancer Res.* **61,** 3308-3313. A comparison of the $\alpha_6\beta_1$ binding sequences reported to date reveals no consensus sequence that acts as an $\alpha_6\beta_1$ binding motif. Furthermore, our newly identified T1 sequence in CCN1 does not exhibit any sequence similarity to these $\alpha_6\beta_1$ binding peptides. Thus, integrin $\alpha_6\beta_1$, like $\alpha_M\beta_2$, is capable of recognizing a broad range of binding sequences. At present, whether these vastly different peptide sequences bind to the same or different sites in $\alpha_6\beta_1$ remains to be determined. Nonetheless, given that integrin $\alpha_6\beta_1$ has been implicated in a multitude of biological processes, different $\alpha_6\beta_1$ binding sequences may interact with distinct coordination sites

within the $\alpha_6\beta_1$ ligand binding pocket to induce different signaling pathways that mediate disparate biological activities.

Please amend and replace paragraph [0065] with the following paragraph:

To define the CCN1 structural domain that interacts with integrin $\alpha_6\beta_1$, we expressed each of these three domains in insect cells via a baculovirus vector (Fig. 1A). To enhance protein secretion, we employed the pMelBac B vector (Invitrogen Incorp.) to provide an N-terminal honeybee melittin secretion signal peptide. To produce the coding sequences for domain I (IGFBP), domain II (VWC), and domain III (TSP1), we used the following respective primer sets for PCR upon the CCN1 cDNA:

5'-CGCGGATCCGGCGCTCTCCACCTGC-3' **(SEQ ID NO: 6)**

and

5'-GGAATTCCCTCTGCAGATCCCTTTCAGAGCGG-3'

(SEQ ID NO: 7)

5'-CGCGGATCCGGCTCAGTCAGAAGGCAGAC-3'

(SEQ ID NO: 8) and

5'-GGAATTCCCAGGAAGCCTCTTCAGTGAGCTGCC-3'

(SEQ ID NO: 9)

5'-CGCGGATCCGGTCTTTGGCACC-3' **(SEQ ID NO: 10)** and

5'-GGAATTCCCTTTTAGGCTGCTGTACACTGGTTGTC-3'

(SEQ ID NO: 11)

Please amend and replace paragraph [0071] with the following paragraph:

We employed another systematic screening strategy to pinpoint the integrin $\alpha_6\beta_1$ binding site in CCN1. A series of overlapping peptides (Table 1) that covers the entire first three

domains of CCN1 was prepared by expression of the peptides as fusion proteins linked to GST. The coding sequences for various peptides (Fig. 3) were amplified by polymerase chain reactions (PCR) upon the CCN1 cDNA as template. Primers used corresponded to the appropriate coding sequences and contain the *Bam*HI and *Eco*RI restriction sites for cloning. For example, the following primers were used to generate the T1 peptide coding sequence:

5'-CGGGATCCGCGGGCCAGAAATGCATCGTT-3'

(SEQ ID NO: 12) and

5'-CCGGAATTCCGCTCTTGGAGCACTGGGACC-3'

(SEQ ID NO: 13)

Please amend and replace paragraph [0076] with the following paragraph:

To determine which residues within the T1 sequence are critical determinants for $\alpha_6\beta_1$-dependent cell adhesion, we prepared a series of GST-peptide fusions that carries the T1 backbone with single or double alanine substitutions at residues conserved among CCN1, CCN2, and CCN3, and tested their abilities to support cell adhesion. To generate site-directed alanine substitutions for the T1 peptide (Fig. 6), synthetic oligonucleotides were annealed for PCR to generate the appropriate coding sequences and cloned into pGEX-4T-2. The following primers were used to prepare the coding sequence for the T1 sequence and cloned into pGEX-4T-2:

5'-GATCCGGTCAAAAATGTATTGTTCAAACTACTTCTTGG TCTCAATGCTCTAAATCTGG-3' **(SEQ ID NO: 14)** and

5'-AATTCCAGATTTAGAGCATTGAGACCAAGAAGTAGTA GTTTGAACAATACATTTTTGACCG-3' **(SEQ ID NO: 15)**

To create the mutant peptides, relevant codons were changed to either GCA or GCT for alanine.

Please amend and replace paragraph [0077] with the following paragraph:

As shown in Fig. 6, alanine substitutions at residues K226, I228, or Q230 did not affect the peptide's ability to support cell adhesion. While single mutation at either T231 or T232 resulted in partial reduction of cell adhesion, combined alanine substitutions of T231 and T232 completely abolished the ability of T1 to support cell adhesion. In addition, single substitutions in W234, S235, S238, or K239 resulted in >90% loss of T1 activity. When mutations in W234 and K239 were combined, cell adhesion was completely obliterated. These results indicate that TTSWSQCSKS **(SEQ ID NO: 1)** is the core sequence in T1 for mediating $\alpha_6\beta_1$ binding. These data also explain the inability of the T2 peptide, which overlaps with the T1 peptide but lacks the TT residues of the core sequence, to inhibit $\alpha_6\beta_1$-dependent cell adhesion.

Please amend and replace paragraph [0083] with the following paragraph:

Mouse CCN1 engineered with a C-terminal FLAG tag was constructed by using the primer sets upon the mouse *Ccn1* cDNA as template

F1: 5'-CGCAATTGGAAAAGGCAGCTCACTGAAGAGGC-3' **(SEQ ID NO: 16)** and

F2: 5'-CCGGAATTCCTACTTGTCATCGTCATCCTTGTAGTCGTCCCTGAA CTTGTGGATGTCATTG-3' **(SEQ ID NO: 17)**,

thus yielding a PCR product containing the last codon of *Ccn1* followed by the FLAG tag coding sequence and a stop codon. The PCR product was double digested with *Nco*I and *Eco*RI, and ligated into pre-cut vector to substitute the *Nco*I, *Eco*RI-digested fragment of the full-length mouse *Ccn1* cDNA in pBlueBac4.5 vector (Grzeszkiewicz, T. M., Kirschling, D. J., Chen, N., and Lau, L. F. (2001) *J. Biol. Chem.* **276,** 21943-21950). For consistency, WT CCN1 and all mutants used in this study were similarly constructed with the same N-terminal secretory signal and C-terminal FLAG epitope tag.

Please amend and replace paragraph [0084] with the following paragraph:

Consistent with the inability of alanine substitution mutants to be secreted, the T1 region of CCN1 appeared highly sensitive to perturbation. Substitutions in the critical T1 residues (W234, S235, S238, K239) with nearly any amino acid resulted in drastic changes in the predicted protein structure. However, we found one mutation, namely K239E that did not elicit a predicted conformational change. To test the efficacy of this mutation in disrupting interaction with $\alpha_6\beta_1$, we constructed a fusion protein with GST linked to the T1 peptide where the T1 peptide carried the K239E mutation (GQKCIVATTSWSQCS*E*S) **(SEQ ID NO: 18)**.

Please amend and replace paragraph [0088] with the following paragraph:

We constructed the K239E single mutation in the context of full length CCN1, designated SM (Fig. 9A) using site-directed mutagenesis using a two-step PCR procedure as described in Koskinen, P., Lehvaslaiho, H., MacDonald Bravo, H., Alitalo, K., and Bravo, R. (1990) *Oncogene.* **5,** 615-618. The internal primer sets were

5'-GTCTTGGTCCCAGTGTTCCGAGAGCTGCGG-3' **(SEQ ID NO: 19)** and

5'-CACTGGGACCAAGACGTGGTCTGAACGATGC-3'**(SEQ ID NO: 20)**.

This construct also created a silent mutation at C237, thereby providing a screening marker by eliminating a BSP12861 restriction site. The outside primers used in PCR were F1 and F2 as described in Example A_1 above. SM was constructed using the mouse *Ccn1* cDNA with a FLAG tag as PCR template.

Please amend and replace paragraph [0089] with the following paragraph:

We also constructed the DM coding sequence, which has disrupted heparin binding sites H1 and H2 as shown in Fig. 9A. These mutations changed H1 from KGKKCSKTKKSPEPVR **(SEQ ID NO: 21)** to AGAACSATAKSPEPVR **(SEQ ID NO: 22)** and H2 from FTYAGCSSVKKYRPKY **(SEQ ID NO: 23)** to FTYAGCSSVAAYAPKY **(SEQ ID NO: 24)**, in the same background as CCN1. We used the DM coding sequence to generate the DM construct with a FLAG tag using the procedure described above. We created the TM sequence using DM as a

template to generate the K239E mutation using the methods described above. All constructs were confirmed by direct sequence analysis.

Please amend Abstract and replace paragraph [00101] with the following paragraph:

The angiogenic inducer CCN1 (cysteine-rich 61, CYR61), a secreted matricellular protein of the CCN family, is a ligand of multiple integrins including $\alpha_6\beta_1$. Previous studies have shown that CCN1 interaction with integrin $\alpha_6\beta_1$ mediates adhesion of fibroblasts, endothelial cells, and smooth muscle cells, as well as migration of smooth muscle cells. Recently, we have reported that CCN1-induced tubule formation of unactivated endothelial cells is also mediated through integrin $\alpha_6\beta_1$. In this study, we demonstrate that human skin fibroblasts adhere specifically to the T1 sequence (GQKCIVQTTSWSQCSKS) **(SEQ ID NO: 2)** within domain III of CCN1, and this process is blocked by anti-a_6 and anti-b_1 monoclonal antibodies. Alanine substitution mutagenesis of the T1 sequence further defines the sequence TTSWSQCSKS **(SEQ ID NO: 1)** as the critical determinant for mediating $\alpha_6\beta_1$-dependent adhesion. Soluble T1 peptide specifically inhibits fibroblast adhesion to CCN1 in a dose-dependent manner. Furthermore, T1 also inhibits cell adhesion to other $\alpha_6\beta_1$ ligands including CCN2 (CTGF), CCN3 (NOV), and laminin, but not to ligands of other integrins. In addition, T1 specifically inhibits $\alpha_6\beta_1$-dependent tubule formation of unactivated endothelial cells in a CCN1-containing collagen gel matrix. To confirm that T1 binds integrin $\alpha_6\beta_1$ directly, we perform affinity chromatography and show that integrin $\alpha_6\beta_1$ is isolated from an octylglucoside extract of fibroblasts on T1-coupled Affi-gel. Taken together, these findings define the T1 sequence in CCN1 as a novel binding motif for integrin $\alpha_6\beta_1$, and form the basis for the development of peptide mimetics to examine the functional role of $\alpha_6\beta_1$ in angiogenesis.